Bioresource Technology 368 (2023) 128319

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Development of a 5-aminolevulinic acid feeding strategy capable of enhancing *Haematococcus pluvialis* biomass, astaxanthin, and fatty acid yields

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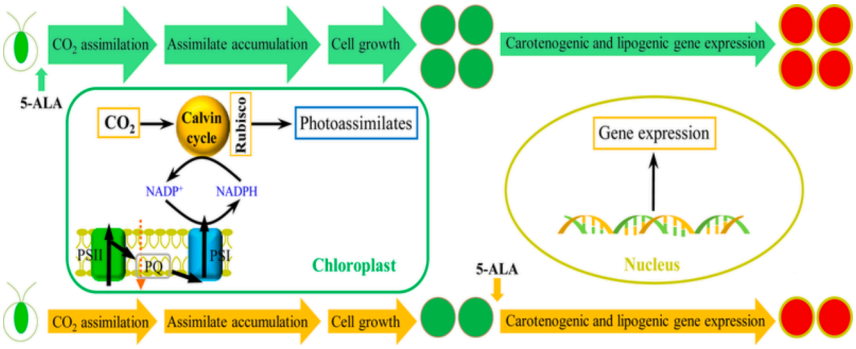
HIGHLIGHTS

- A 5-ALA feeding strategy was established to enhance astaxanthin yield in *H. pluvialis*.
- Feeding 4 μM 5-ALA on day 1 caused the greatest increase in astaxanthin yield.
- Feeding 5-ALA to cells at an early stage of growth increased biomass accumulation.
- 5-ALA improved biomass production by enhancing photosynthetic CO_2 assimilation.
- 5-ALA improved astaxanthin and fatty acid yields by enhancing biomass production.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
5-aminolevulinic acid
Biomass
Astaxanthin
Haematococcus pluvialis
Stimulator

ABSTRACT

Effective inducers play essential roles in the regulation of cell growth and astaxanthin production in *Haematococcus pluvialis*. Here, a novel 5-aminolevulinic acid (5-ALA) feeding strategy was developed and found to enhance *H. pluvialis* biomass, fatty acid, and astaxanthin yields. Specifically, 5-ALA feeding (4 μM) on day 1 caused respective 23.8 %, 24.8 %, and 20.3 % increases in biomass, fatty acid, and astaxanthin yields. The observed enhancement of biomass accumulation associated with the provision of 5-ALA during the early stages of growth was attributable to enhanced photosynthetic carbon assimilation. This increased biomass accumulation, in turn, contributed to the measured increases in both fatty acid and astaxanthin yields. Overall, these results provide new insight into the importance of photosynthetic carbon assimilation as a determinant of biomass accumulation and a regulator of both fatty acid and astaxanthin production, while offering an effective strategy capable of accelerating astaxanthin production by *H. pluvialis* in commercial agricultural contexts.

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https://doi.org/10.1016/j.biortech.2022.128319
Received 4 October 2022; Received in revised form 7 November 2022; Accepted 8 November 2022
Available online 12 November 2022

1. Introduction

Astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione) is a carotenoid that is naturally produced by microalgae and certain species of fish, birds, and crustaceans (Hussein et al., 2006). The antioxidant activity of astaxanthin is 500-fold higher than that of vitamin E, and relative to β-carotene it has a 38-fold higher potential to terminate chain reactions responsible for the generation of free radicals such that it is commonly applied in the food, cosmetic, pharmaceutical, and neutraceutical industries (Oslan et al., 2021). *Haematococcus pluvialis* is a species of spherical unicellular green biflagellate oleaginous cells ~ 30 μm in diameter capable of producing large quantities of astaxanthin (Aflalo et al., 2007; Mehariya et al., 2020). These cells are exploited to produce astaxanthin through a two-stage process, initially growing rapidly and amassing substantial biomass before being exposed to stressful environmental conditions that shift the metabolic activity of these cells to favor carotenogenesis (Wang et al., 2014). Both green algae and higher plants share the initial phase of the astaxanthin biosynthesis pathway to the formation of β-carotene, after which species-specific oxygenation reactions ultimately govern the conversion of β-carotene into astaxanthin (Li et al., 2019).

Many studies of *H. pluvialis* cultivation have been conducted in an effort to optimize biomass accumulation and astaxanthin production strategies. Current studies highlight three areas for potential improvement, including the selection of appropriate *H. pluvialis* strains suited to large-scale culture, the design of more efficient production processes, and the selection of appropriate environmental induction conditions (Zhang et al., 2009). Astaxanthin accumulation is generally induced by establishing stressful environmental conditions (Park et al., 2014), including low levels of nutrient availability, high salt levels, high temperatures, or high light conditions (Ambati et al., 2014; Sarada et al., 2002). These stressors induce physiological changes and corresponding transcriptomic alterations in exposed cells (Zhao et al., 2020; Steinbrenner and Linden, 2003).

Basic carbon skeletons serve as essential precursors for astaxanthin biosynthesis, and the exogenous application of certain forms of organic carbon may thus represent an effective means of enhancing *H. pluvialis* astaxanthin accumulation. Indeed, several prior reports have shown that adding sources of organic carbon including acetate, oxaloacetate, polyalcohol, and sugar can augment astaxanthin production (Azizi et al., 2019; Du et al., 2021; Yu et al., 2022; Zhang et al., 2019; Zhang et al., 2020). Exogenous organic carbon sources could promote astaxanthin accumulatoin by increasing the biosynthesis of substrates (Yu et al., 2022; Zhang et al., 2020). *H. pluvialis* astaxanthin yields also benefit from the application of exogenous plant hormones or analogs thereof, including melatonin, salicylic acid, methyl jasmonate, and gibberellin (Lu et al., 2010; Gao et al., 2012; Xing et al., 2022). These accelerants are probably involved in the production of reactive oxygen species or play key roles in mediating the signaling network associated with astaxanthin metabolism in *H. pluvialis* (Zhang et al., 2022). In order to further enhance astaxanthin production, new accelerants are needed to be selected and investigated.

The plant growth regulator 5-aminolevulinic acid (5-ALA) serves as a precursor that is essential for the biosynthesis of vital tetrapyrrole compounds including chlorophyll, heme, and vitamin B12 (Senge et al., 2014). The application of 5-ALA can effectively promote the growth and development of plants, rendering them more resistant to stress exposure (Wu et al., 2019), with 5-ALA-treated plants being better able to tolerate high levels of salinity or other environmental stressors (Wu et al., 2019; Wang et al., 2021). The present study was conducted to explore the ability of 5-ALA to promote biomass and astaxanthin accumulation in *H. pluvialis* and to clarify the underlying regulatory mechanisms for the first time. The clarification of the benefits of exogenous 5-ALA administration may aid in the future optimization of astaxanthin production in the context of large-scale outdoor *H. pluvialis* cultivation.

2. Material and methods

2.1. Microalgal culture

H. pluvialis, a freshwater green microalga, was initially cultured to an appropriate density in MCM medium (pH 7.0), with exposure of 500 mL Erlenmeyer flasks containing 300 mL microalgal cultures to 200 μmol photons m^{-2} s^{-1} illumination. Following a 15-day culture interval, these *H. pluvialis* cells had reached a stationary phase of growth, and on day 16 the light levels were increased to 500 μmol photons m^{-2} s^{-1} to induce astaxanthin production. The exogenous administration of 5-ALA (0, 1, 2, 4, or 6 μM) (Sigma, MO, USA) to these cultures was performed on either day 1 or day 16. All culture was performed at 25 ± 1 °C with a 14L/10D photoperiod. Culture flasks were shaken by hand 4–6 times per day to prevent the sticking of cells. All of the experiments were carried out in triplicate.

2.2. Cell density, dry weight, and astaxanthin analyses

H. pluvialis cells were evaluated with an inverted optical microscope (37XB, Shanghai, China). Samples were counted by harvesting a 20 μL aliquot from these flasks and repeating these analyses in triplicate. For dry weight measurements, cells were centrifuged (10 min, 3,600 rpm) and the pellet was frozen at −80 °C for 12 h, vacuum-dried for 48 h, and weighed. Total astaxanthin was extracted and measured as reported previously by Liu et al. (2002).

2.3. Fatty acid analyses

Fatty acid transesterification was performed using a 2 % H_2SO_4-methanol solution as reported previously by Zhang et al. (2021). The analysis was carried out on a capillary column (30 m × 0.32 mm × 0.25 μm, Omegawax^{TM}320) using a gas chromatograph (GC112A, Shanghai, China). The column temperature was raised from 60 °C to 150 °C at a rate of 20 °C min^{-1}, and it was then kept at this temperature for 2 min. Following that, the temperature was raised to 265 °C at a rate of 4 °C/min. The flame ionization detector and the injector were both set to a temperature of 270 °C. Chromatography was employed to identify fatty acid methyl esters using real standards (methyl heptadecanoate) (Sigma, MO, USA). The fatty acid content was determined by comparing the area of each peak to the area of a standard C17 amount.

2.4. Gene expression analyses

After 1, 7, and 21 days of incubation, gene expression was analyzed using Real-time quantitative PCR (qPCR). Total algal RNA was extracted using a Hi-Pure Plant RNA Mini Kit (Magen, Guangzhou, China). First-strand cDNA synthesis was performed with a TransScript One-Step gDNA Removal and cDNA synthesis SuperMix (TransGen, Beijing, China). All qPCR analyses were performed in triplicate using a Light Cycler 480 instrument (Roche, Basel, Switzerland) and the TransStart Green qPCR Super-Mix Kit (TransGen, Beijing, China). Thermocycler settings were as follows: 95 °C for 1 min; 50 cycles of 95 °C for 10 s, and 60 °C for 30 s. Actin served as a normalization control, and the $2^{-\Delta\Delta CT}$ method was used to quantify relative gene expression (Livak and Schmittgen, 2001).

2.5. Chlorophyll fluorescence analyses

Chlorophyll *a* fluorescence transients and an energy pipeline model of phenomenological fluxes were analyzed at room temperature in *H. pluvialis* cells that had been adapted to the dark for 15 min using a Handy PEA fluorometer (Hansatech, Norfolk, UK). Chlorophyll fluorescence was induced by exposing cells to red light (3000 μmol m^{-2} s^{-1}) using an array consisting of three light-emitting diodes (LEDs) with peak light release at 650 nm.

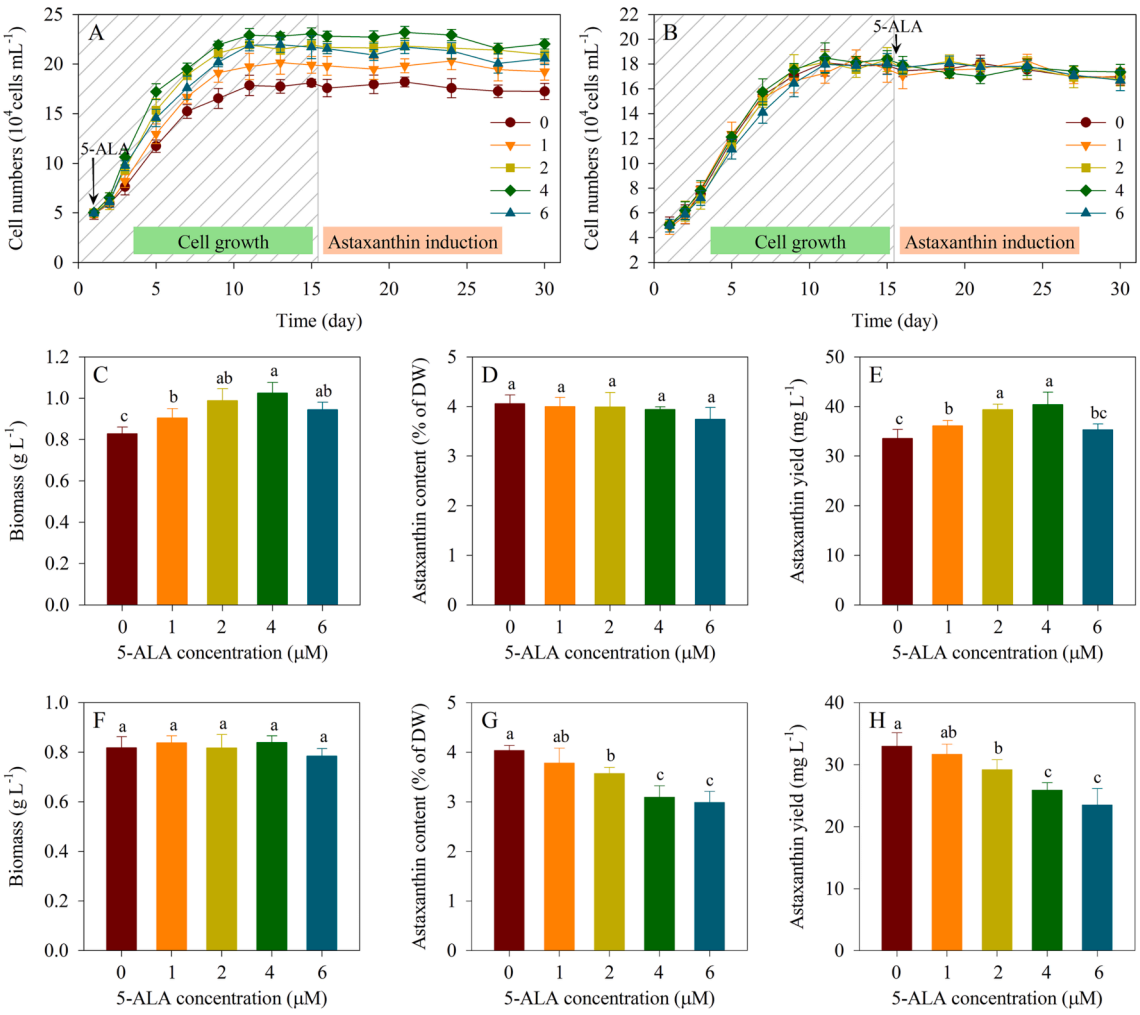

Fig. 1. The impact of exogenous 5-ALA administration on *H. pluvialis* cell density (A, B), biomass (C, F), and astaxanthin production (D, E, G, H). (A, C, D, E) 5-ALA feeding on day 1; (B, F, G, H) 5-ALA feeding on day 16. Different lowercase letters indicate significant difference between the treatments with various concentrations of 5-ALA at $P < 0.05$, the same as this in other figures.

2.6. Enzymatic analyses

The extraction of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was conducted as reported previously by Zhang et al. (2021). Rubisco activity was analyzed in a mixture of 0.5 mM ribulose-1,5-bisphosphate, 25 mM $KHCO_3$, 3.5 mM ATP, 20 mM $MgCl_2$, 100 mM Bicine, 5 mM phosphocreatine, 5 units 3-phosphoglyceric phosphokinase, 5 units glyceraldehydes-3-phosphate dehydrogenase, 17.5 units creatine phosphokinase and 0.25 mM NADH. NADH oxidation was monitored at 340 nm using a U-2900 spectrophotometer (Hitachi, Tokyo, Japan).

2.7. Photosynthetic and respiratory rate analyses

H. pluvialis photosynthetic and respiratory rates were analyzed with a Clark-type O_2 electrode (Hansatech, Norfolk, UK).

2.8. Statistical analysis

Differences among *H. pluvialis* cultures exposed to different 5-ALA treatment conditions were analyzed via a least-significant difference (LSD) test using SPSS 16.0 (IBM, NY, USA). $P < 0.05$ was the significance threshold.

3. Results and discussion

3.1. The impact of 5-ALA feeding on H. pluvialis cell growth and astaxanthin production

In this study, a two-stage cultivation approach was used to achieve robust astaxanthin production by *H. pluvialis*. Initially, cells were cultured from days 1–15 (cell growth phase) under favorable conditions to promote optimal growth. When these cells reached the stationary phase of growth, they were then exposed to high light conditions from days 16–30 (astaxanthin induction phase) to promote the accumulation of astaxanthin.

Under control (0 μM 5-ALA) conditions, *H. pluvialis* cell numbers rose from 4.3×10^4 to 17.7×10^4 cells/mL during the cell growth phase, after which they remained largely stable during the astaxanthin induction phase (Fig. 1A). Exogenous 5-ALA administration on day 1 resulted in significant increases in microalgal cell numbers relative to these control conditions such that on day 15, the maximum cell density in cultures that had been supplemented with 4 μM 5-ALA was 27.3 % higher than that in the control group. However, lower *H. pluvialis* cell density was observed when cells were treated on day 1 with 6 μM 5-ALA relative to treatment with 4 μM 5-ALA (Fig. 1A). *H. pluvialis* cell numbers remained relatively stable throughout the astaxanthin induction phase irrespective of the 5-ALA dose administered on day 1. When the supply of nutrients including phosphorus and nitrogen is insufficient to

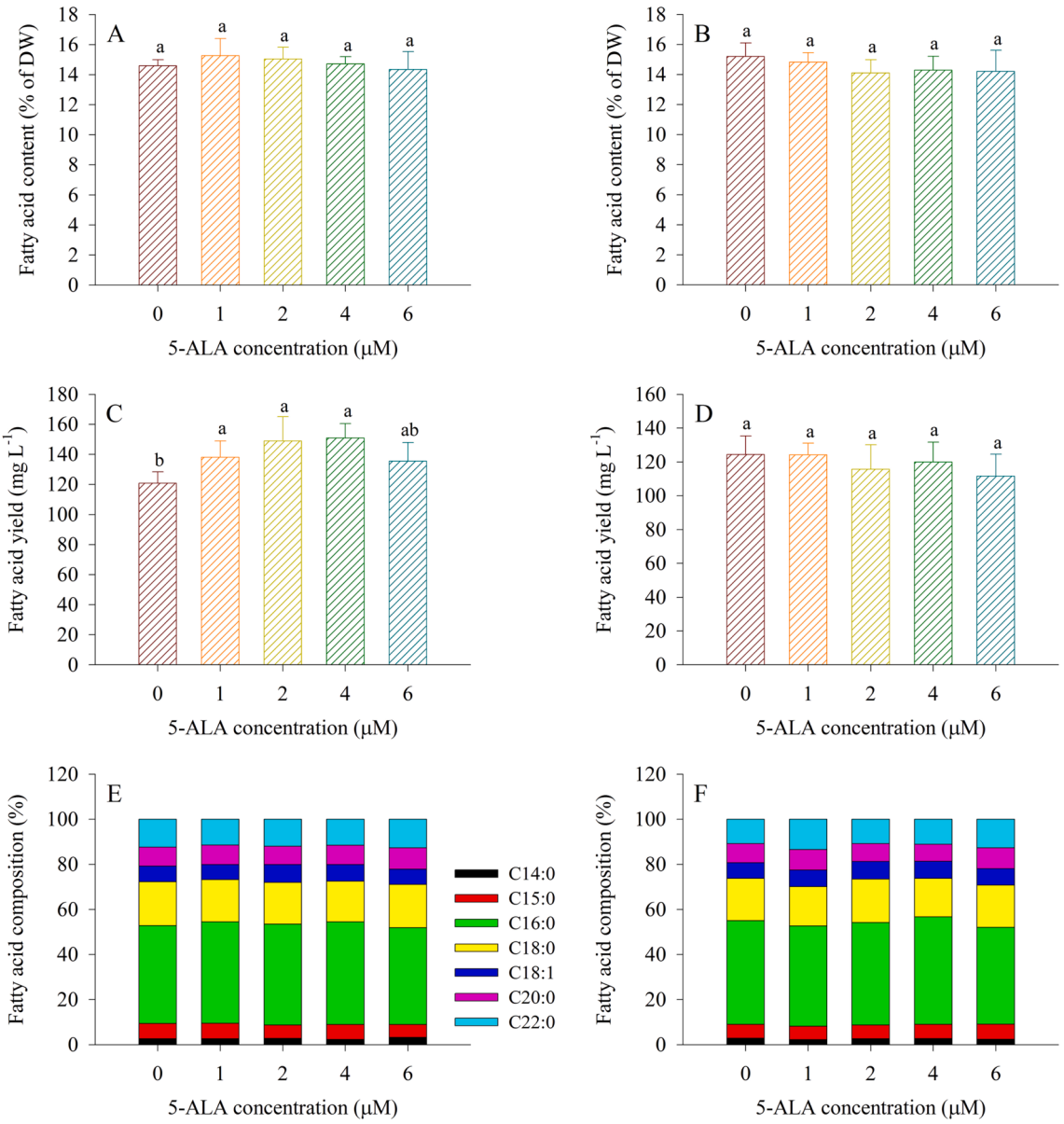

Fig. 2. The impact of exogenous 5-ALA administration on the biosynthesis of fatty acids (A, B, C, D) and fatty acid composition (E, F) in *H. pluviualis*. (A, C, E) 5-ALA feeding on day 1; (B, D, F) 5-ALA feeding on day 16.

facilitate microalgal growth, these cells ultimately enter the stationary phase (Zhang et al., 2022), thus explaining the lack of any substantial increases in *H. pluvialis* cell numbers after 11 days of culture (Fig. 1A). The addition of exogenous 5-ALA on day 16 at the start of astaxanthin induction failed to result in any further increases in cell yields (Fig. 1B).

As a result of the higher observed cell density under these conditions, 5-ALA feeding on day 1 led to an increase in *H. pluvialis* biomass (Fig. 1C). The maximum biomass at the end of the 30-day culture period was observed in the 4 μM 5-ALA treatment group. Relative to the control group, biomass accumulation in the groups treated with 1, 2, 4, and 6 μM 5-ALA on day 1 were increased by 9.3 %, 19.5 %, 23.8 %, and 14.1 %. This is in accordance with previous reports that 5-ALA promotes cell growth and biomass accumulation in *Chlorella pyrenoidosa* (Li et al., 2022). Treatment with 5-ALA on day 16 failed to impact biomass accumulation at the end of the 30-day culture period owing to the lack of any change in cell density (Fig. 1F).

While treatment with different concentrations of 5-ALA on day 1 did not increase *H. pluvialis* astaxanthin content at the end of the 30-day incubation period (Fig. 1D), it did increase astaxanthin yields as a result of the increase in cell density (Fig. 1E). The maximum astaxanthin yield at the end of the 30-day culture period was observed in the group treated with 4 μM 5-ALA, consistent with the cell density and biomass results. Relative to the control group, astaxanthin yields in the groups treated with 1, 2, 4, and 6 μM 5-ALA were increased by 7.6 %, 17.2 %, 20.3 %, and 5.2 %. Conversely, 5-ALA administration on day 16 resulted in a significant decrease in astaxanthin content in these *H. pluvialis* cells at the end of the 30-day culture period (Fig. 1G), and astaxanthin yields also fell with increasing 5-ALA concentration under these conditions (Fig. 1H). This suggests that the administration of an appropriate 5-ALA dose at the proper time point is vital to improve microalgal biomass accumulation and astaxanthin production. The present results suggest that supplementing *H. pluvialis* cultures with 4 μM 5-ALA on day 1 of the cell growth period was the optimal tested treatment strategy, providing a means of effectively enhancing biomass and astaxanthin yields.

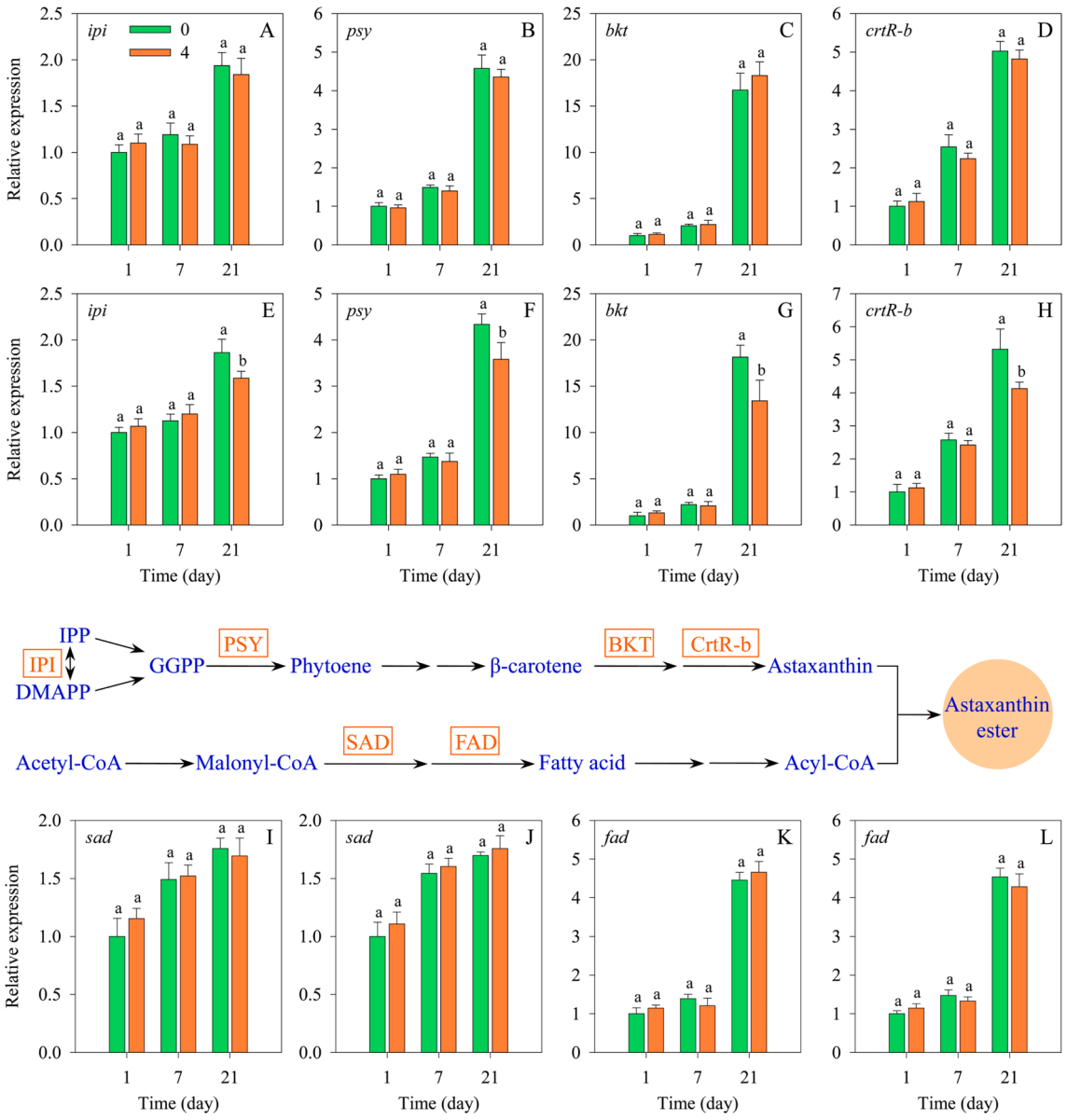

Fig. 3. The impact of exogenous 5-ALA administration on the mRNA level expression of the carotenogenic genes *ipi* (A, E), *psy* (B, F), *bkt* (C, G), and *crtR-b* (D, H) as well as the lipogenic genes *sad* (I, J) and *fad* (K, L) in *H. pluviualis*. (A-D, I, K) 5-ALA feeding on day 1; (E-H, J, L) 5-ALA feeding on day 16.

3.2. The impact of 5-ALA administration on H. pluvialis fatty acid production

The majority of astaxanthin produced in *H. pluvialis* cells incorporates fatty acids, thereby generating astaxanthin esters. This esterification process depends on fatty acid formation and is capable of promoting astaxanthin production by *H. pluvialis* (Chen et al., 2015). The process of astaxanthin esterification can also be impacted by the fatty acid composition within cells, as C16:0 and C18:1 are the fatty acids that are most frequently esterified with astaxanthin (Breithaupt, 2004; Chen et al., 2015).

In line with the astaxanthin results, no significant changes in fatty acid contents were detected on day 30 in *H. pluvialis* cells that had been treated on day 1 with different 5-ALA concentrations (Fig. 2A), whereas the fatty acid yields for these 5-ALA-treated cultures were increased owing to the measured increase in cell density (Fig. 2C). The maximum fatty acid yield was observed in *H. pluvialis* cultures treated with 4 μM 5-ALA on day 1. Relative to control cells, cultures treated with 1, 2, 4, and 6 μM 5-ALA exhibited respective 14.2 %, 23.2 %, 24.8 %, and 12.0 % increases in fatty acid yields. In contrast, the application of 5-ALA on day 16 failed to impact fatty acid content or yields in *H. pluvialis* cultures measured on day 30 at any tested dose level (Fig. 2B, D). As such, these data indicate that treatment on day 1 with 4 μM 5-ALA is the optimal tested approach to increasing *H. pluvialis* fatty acid yields.

Fatty acid composition analyses revealed that C16:0 and C18:0 accounted for the majority (62.9 %) of fatty acids in control *H. pluvialis* cultures (Fig. 2E), and no changes in fatty acid composition were detected at any tested 5-ALA dose level irrespective of whether 5-ALA was added on day 1 or day 16 (Fig. 2F). This suggests that adding 5-ALA on day 1 does not directly alter astaxanthin or fatty acid content within these microalgal cells, but instead significantly enhances astaxanthin and fatty acid yields as a result of corresponding increases in cell density.

3.3. Carotenogenic and lipogenic H. pluvialis gene responses to 5-ALA administration

Next, the expression of important genes involved in the process of

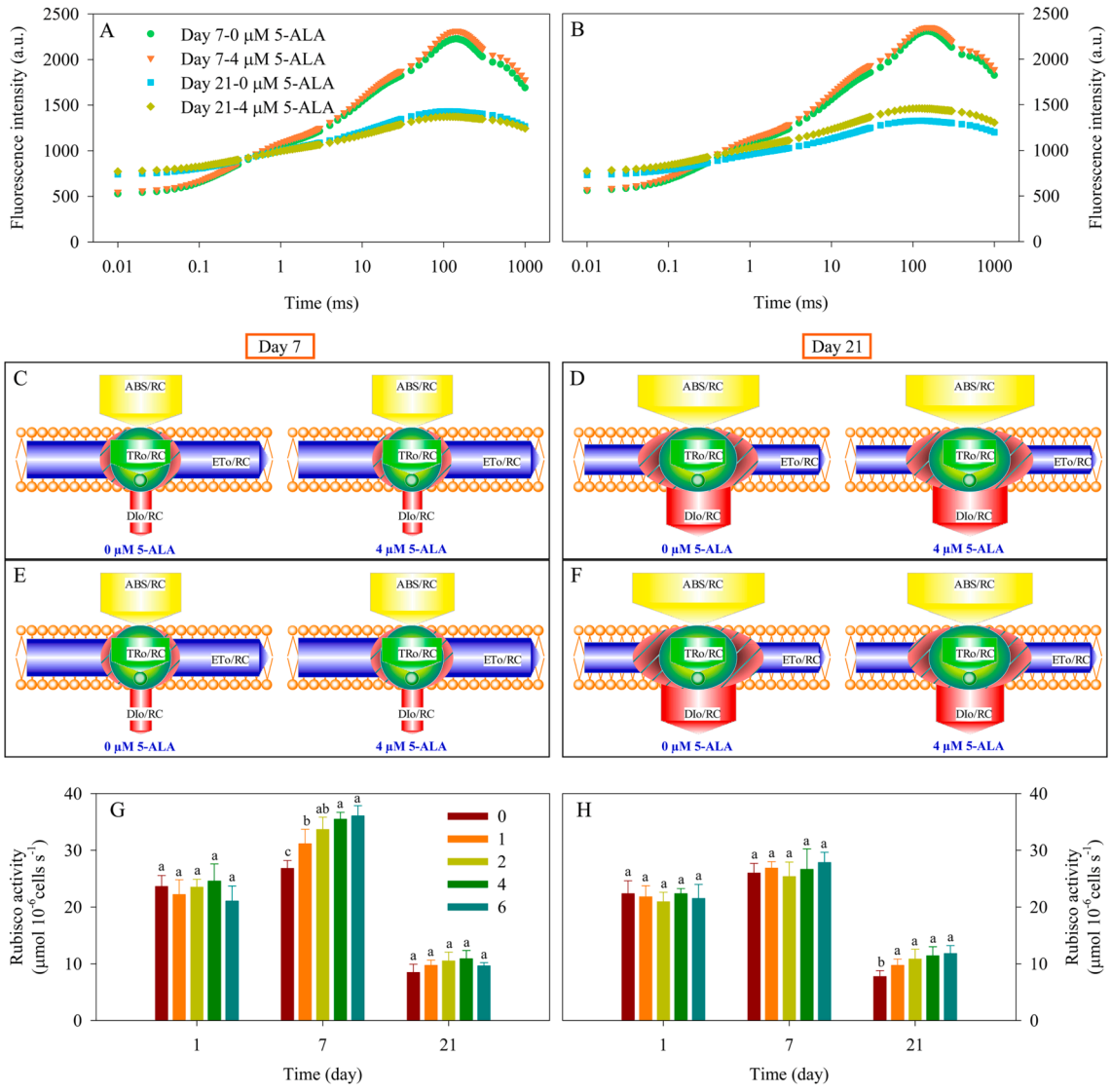

Fig. 4. The impact of exogenous 5-ALA administration on chlorophyll *a* fluorescence transients (A, B), energy pipeline model of phenomenological (per excited cross-section, CSm) fluxes (C-F) and Rubisco activity (G, H) in *H. pluviualis*. (A, C, D, G) 5-ALA feeding on day 1; (B, E, F, H) 5-ALA feeding on day 16.

astaxanthin biosynthesis was analyzed under these culture conditions. Isopentenyl pyrophosphate (IPP) is an important precursor used by *H. pluvialis* cells to synthesize astaxanthin (Han et al., 2013). The *ipi* gene encodes an IPP isomerase responsible for catalyzing conversion between IPP and dimethylallyl diphosphate (DMAPP) (Lichtenthaler, 1999). The rate-limiting carotenoid biosynthesis step in microalgal cells relies on the phytoene synthase (encoded by *psy*)-mediated catalyzation of phytoene biosynthesis from two 20-carbon geranylgeranyl pyrophosphate molecules (Gong and Bassi, 2016; Lu et al., 2020). The *bkt* and *crtR-b* genes respectively encode β-carotene ketolase (BKT) and β-carotene hydroxylase (CrtR-b), which catalyze astaxanthin synthesis from β-carotene (Solovchenko, 2015), and are the rate-limiting enzymes in the process of astaxanthin biosynthesis (Han et al., 2013).

Gradual increases in the expression of *ipi*, *psy*, *bkt*, and *crtR-b* were detected in *H. pluvialis* samples with the prolongation of the culture period gradually with increased incubation time (Fig. 3A-H). No changes in the expression of any of these genes were observed over the course of incubation when cells were treated with 4 μM 5-ALA on day 1 (Fig. 3A-D). However, the administration of 4 μM 5-ALA on day 16 significantly reduced measured *ipi*, *psy*, *bkt*, and *crtR-b* expression on day 21 (Fig. 3E-H). This suggests that the administration of 5-ALA during the cell growth phase of culture failed to impact the astaxanthin biosynthesis process in these *H. pluvialis* cells, whereas adding 5-ALA during the astaxanthin induction phase suppressed this biosynthetic process such that astaxanthin content and yields were reduced (Fig. 1G, H). As abiotic stressors can promote reactive oxygen species (ROS) accumulation (Zhang et al., 2022), astaxanthin biosynthesis has been induced by both exposure to high light and nutrient deficiency conditions (Liu et al., 2016). These ROS levels can drive the more rapid production of astaxanthin by upregulating genes involved in the synthesis of this carotenoid (Lemoine and Schoefs, 2010). As 5-ALA can restore cellular redox homeostasis and antioxidant activity, it can prevent such ROS accumulation (Wu et al., 2019). This thus explains the observed inhibition of astaxanthin biosynthetic gene expression when 5-ALA was added during the astaxanthin induction phase of the *H. pluvialis* culture process (Fig. 3E-H), resulting in decreased astaxanthin content and yields (Fig. 1G, H). As the addition of 5-ALA on day 1 had no adverse impact on astaxanthin biosynthetic gene expression (Fig. 3A-D), this suggests that the provided astaxanthin was fully utilized during the cell growth phase. Further studies will be vital to determine whether the use of even higher 5-ALA concentrations during the cell growth phase can impact the process of astaxanthin biosynthesis.

Key fatty acid biosynthesis-related gene expression patterns were also examined in these microalgal cultures. Stearoyl-ACP-desaturase

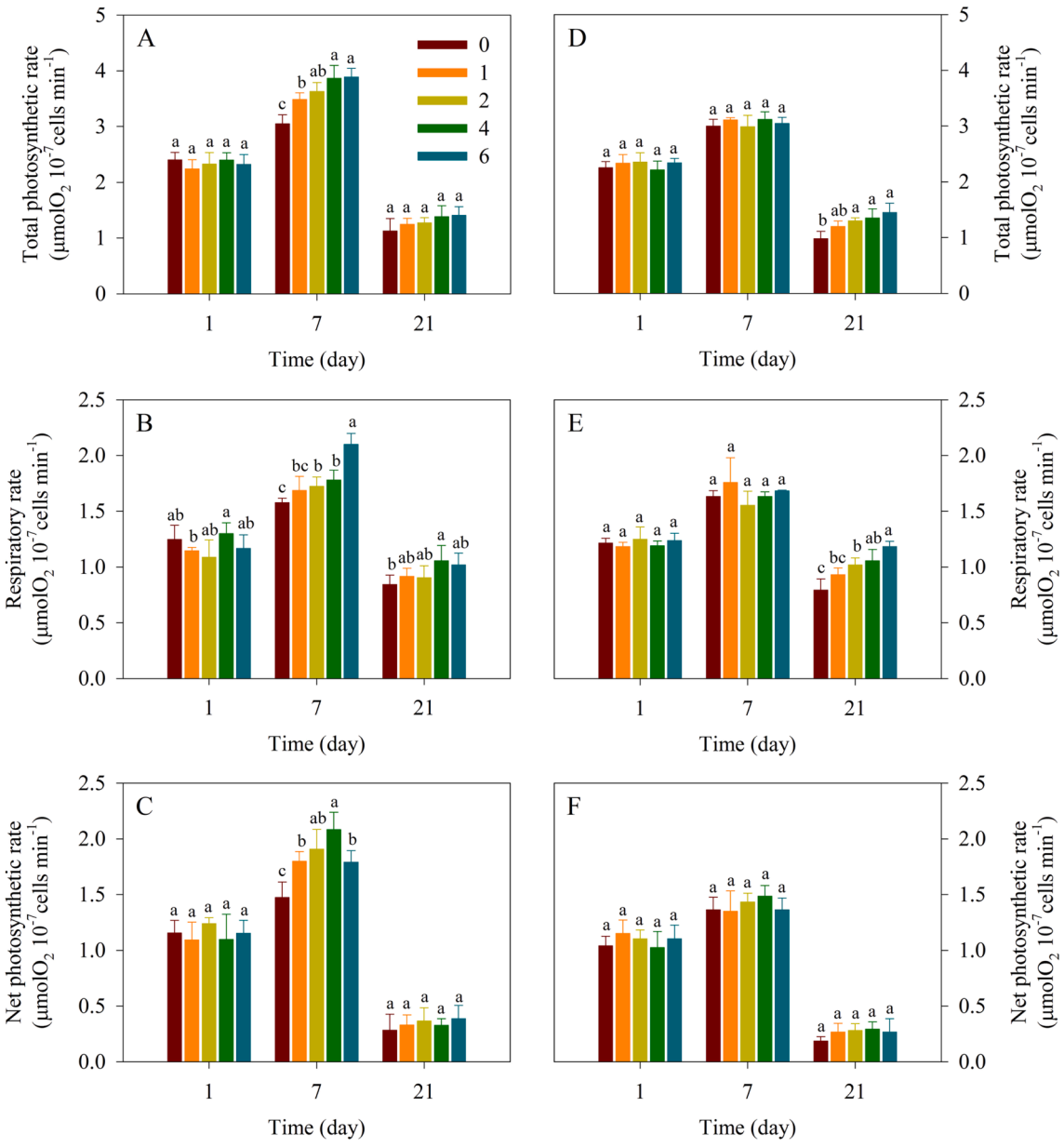

Fig. 5. The impact of exogenous 5-ALA administration on total photosynthetic rate (A, D), respiratory rate (B, E), and net photosynthetic rate (C, F) in *H. pluviualis*. (A, B, C) 5-ALA feeding on day 1; (D, E, F) 5-ALA feeding on day 16.

(SAD) and ω-3 fatty acid desaturase (FAD), respectively encoded by the *sad* and *fad* genes, catalyze long-chain fatty acid assembly during fatty acid biosynthesis (Penfield et al., 2006). No changes in the expression of these lipogenic genes were observed in *H. pluvialis* cultures supplemented with 4 μM 5-ALA on day 1 or day 16 (Fig. 3I-L), consistent with the absence of any observed change in fatty acid content (Fig. 2A, B).

Together, these data support a model in which exogenous 5-ALA administration has no direct promote astaxanthin or fatty acid biosynthesis in *H. pluvialis*. However, providing an appropriate dose of 5-ALA during the optimal culture window can improve astaxanthin and fatty acid yields through the enhancement of cell growth. It has been suggested that *H. pluvialis* has the ability to absorb and utilize organic carbon sources, such as acetate, oxaloacetate, and glycerol (Yu et al., 2022; Zhang et al., 2019; Zhang et al., 2020). Exogenous organic carbon sources can accelerate astaxanthin and fatty acid production by enhancing the biosynthesis of substrates. Several chemicals, such as plant hormones and polyamines, can also promote astaxanthin and fatty acid production (Lu et al., 2010; Gao et al., 2012; Xing et al., 2022). These chemicals are probably involved in the generation of ROS or play key roles in mediating the signaling network associated with astaxanthin and fatty acid biosynthesis in *H. pluvialis*. Therefore, most of these accelerants reported in previous studies can directly facilitate astaxanthin and fatty acid biosynthesis. However, in this study, feeding these *H. pluvialis* cells with 5-ALA on day 1 can facilitate improved cell growth, thereby indirectly increasing astaxanthin and fatty acid production. As such, further research was conducted with the goal of clarifying the mechanisms through which 5-ALA promotes *H. pluvialis* cell growth.

3.4. The mechanisms whereby 5-ALA enhances cell growth to improve astaxanthin and fatty acid yields

Photosynthetic biomass production and respiratory biomass loss, also known as net photosynthetic biomass production, is the primary determinant of microalgal growth productivity (Zhang and Liu, 2016). In plants, photosynthesis, which consists of both the light reaction and the dark reaction (carbon assimilation), is among the most sensitive

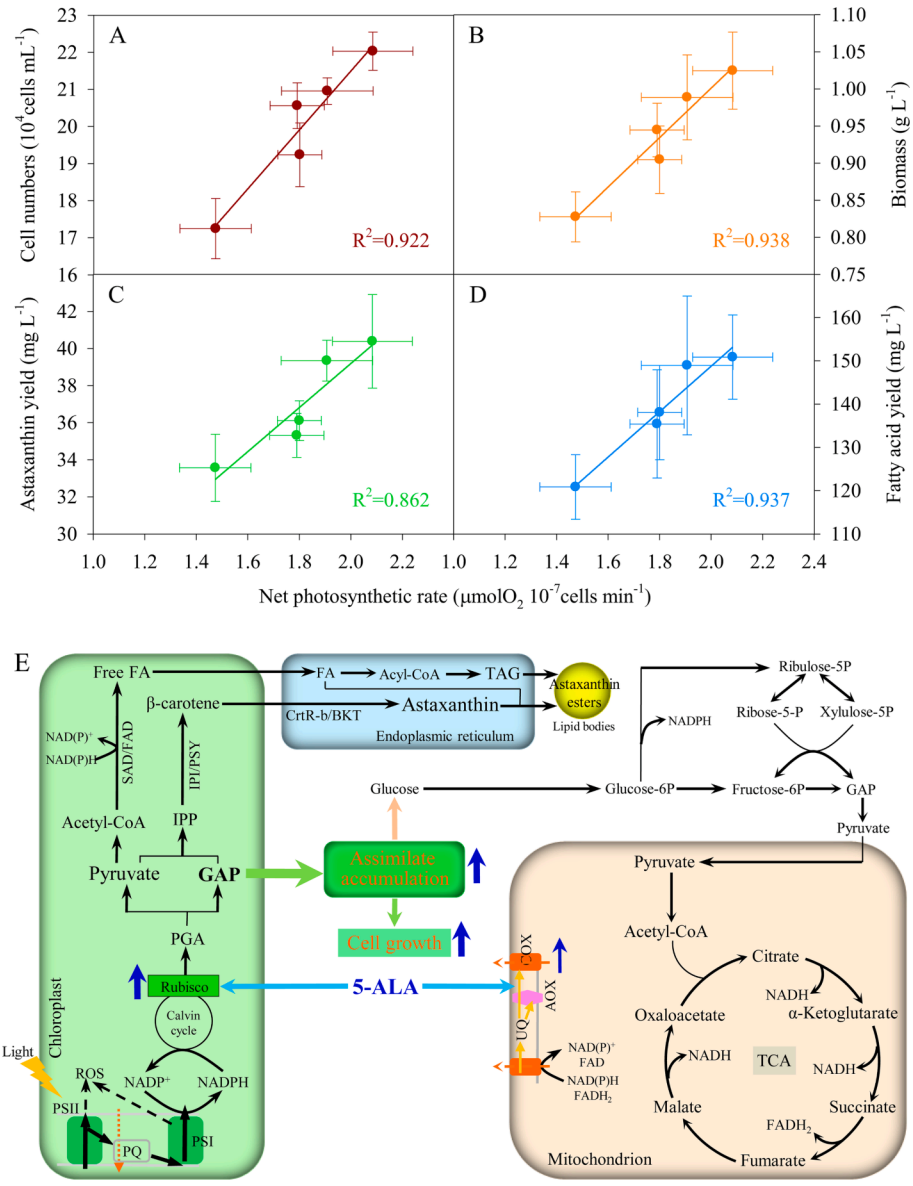

Fig. 6. Correlations between net photosynthetic activity and cell numbers (A), biomass (B), astaxanthin yields (C), and fatty acid yields (D) following treatment on day 1 with the indicated 5-ALA concentrations. (E) Potential mechanisms whereby 5-ALA administration may promote cellular growth and the synthesis of fatty acids and astaxanthin.

processes to the addition of exogenous compounds (Wu et al., 2019; Zhang et al., 2021).

3.4.1. *Exogenous 5-ALA administration has no impact on the photosynthetic light reaction in H. pluvialis*

The chlorophyll *a* fluorescence transient are among the most valuable targets when studying the photosynthetic light reaction (Kalachanis and Manetas, 2010; Zhang et al., 2017), as the shape of this transient is highly sensitive to environmental stress and the addition of exogenous compounds. Chlorophyll *a* fluorescence transient quantification can thus be used to calculate several key parameters associated with the photosynthetic light reaction (Strasser et al., 2000, 2004), allowing for *in vivo* analyses of the energy fluxes associated with absorption, trapping, and electron transport (Strasser et al., 2000; 2004; Zhang et al., 2017).

In this study, 5-ALA administration on day 1 had no impact on the shapes of chlorophyll *a* fluorescence transients (Fig. 4A) or associated phenomenological energy fluxes (Fig. 4C, D) for absorption (ABS/CSm), trapping (TRo/CSm), or electron transport (ETo/CSm) in *H. pluvialis*. Similarly, 5-ALA administration on day 16 had no impact on any of these parameters (Fig. 4B, E, F). As such, exogenous 5-ALA does not affect the photosynthetic light reaction in *H. pluvialis*.

3.4.2. *5-ALA administration enhances photosynthetic carbon assimilation in H. pluviualis*

The ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme plays an essential role in the process of photosynthetic carbon assimilation (Eilenberg et al., 1998). In many plants, low 5-ALA concentrations have been posited to function as signaling molecules that regulate diverse cellular metabolic processes (Wu et al., 2019; Wang et al., 2021). Wang et al. (2018) reported that treatment with 5-ALA led to the optimization of photosynthetic carbon assimilation. Here, significant increases in Rubisco activity were detected in *H. pluvialis* cultures treated on day 1 with different 5-ALA concentrations (Fig. 4G). On day 7, for example, Rubisco activity in the 1, 2, 4, and 6 μM 5-ALA treatment groups was increased by 16.2 %, 25.6 %, 32.3 %, and 34.5 % relative to the control group (Fig. 4G). The administration of different 5-ALA concentrations on day 16 similarly enhanced Rubisco activity in

these cells (Fig. 4H), with a dose-dependent increase in Rubisco activity measured on day 21 in response to 5-ALA treatment. These data indicate that exogenous 5-ALA administration can enhance photosynthetic activity through increases in the dark reaction rather than the light reaction.

3.4.3. Analyses of correlations between net photosynthetic rate and cell growth, astaxanthin yield, and fatty acid yield

During the cell growth phase, the total photosynthetic rate rose before subsequently declining during the astaxanthin induction phase (Fig. 5A, D). The administration of different 5-ALA concentrations on day 1 led to significant increases in the *H. pluvialis* total photosynthetic rate (Fig. 5A), with cultures treated with 1, 2, 4, and 6 μM 5-ALA respectively exhibiting 14.3 %, 19.0 %, 26.7 %, and 27.5 % increases in these rates on day 7 relative to the control group (Fig. 5A). The total photosynthetic rate similarly rose in response to the addition of different 5-ALA concentrations on day 16 (Fig. 5D), resulting in dose-dependent increases in these rates with rising 5-ALA concentrations on day 21. This suggests that exogenous 5-ALA administration enhanced carbon assimilation (Fig. 4), thus improving photosynthetic activity in these *H. pluvialis* cells (Fig. 5A, D). Moreover, 5-ALA is not stable in solution and its degradation rate strongly depends on temperature and pH (Kaliszewski et al., 2007). At pH 7.4 the 5-ALA lost almost 90 % of the initial activity during 5 days of storage at 4 °C, thus explaining the lack of any substantial increases in *H. pluvialis* photosynthetic activities after 21 days of culture when supplemented with different 5-ALA concentrations on day 1 (Fig. 4G, 5A).

Given that 5-ALA serves as a key substrate for the biosynthesis of heme (Wu et al., 2019), which is required for the activity of cytochrome *c* activity in the mitochondrial respiratory electron chain (Gonzalez et al., 2000), cellular respiratory rates rise in response to exogenous 5-ALA supplementation (Wang et al., 2005). Consistently, *H. pluvialis* respiratory rates rose when cultures were treated with different 5-ALA concentrations on day 1 (Fig. 5B). On day 7, relative to the control group, groups treated with 1, 2, 4, and 6 μM 5-ALA exhibited 7.0 %, 9.3 %, 12.9 %, and 33.2 % increases in measured respiratory rate values (Fig. 5B). The administration of 5-ALA on day 16 also significantly enhanced *H. pluvialis* respiratory rates (Fig. 5E), with measured respiratory rate values on day 21 rising with increasing 5-ALA concentration levels (Fig. 5E).

Much like the total photosynthetic rate, the net photosynthetic rate initially rose during the cell growth phase before declining during the astaxanthin induction phase (Fig. 5C, F). The exogenous administration of 5-ALA on day 1 resulted in a net increase in the *H. pluvialis* net photosynthetic rate (Fig. 5C), with this rate being highest in culture flasks treated with 4 μM 5-ALA. On day 7 of the study, net photosynthetic rates in culture flasks exposed to 1, 2, 4, and 6 μM 5-ALA were 22.2 %, 29.4 %, 41.4 %, and 21.5 % higher relative to the control group (Fig. 5C). As the net photosynthetic rate is defined as the difference between total photosynthetic rate and respiration rate, the administration of 6 μM 5-ALA resulted in a reduction in the measured net photosynthetic rate as compared to the 4 μM 5-ALA fed group (Fig. 5C), given that the respiratory rate increased under these conditions (Fig. 5B). The exogenous administration of 5-ALA on day 16 failed to impact the net photosynthetic rate in these *H. pluvialis* cultures (Fig. 5F), as the total photosynthetic rate and respiration rate rose to a similar degree (Fig. 5D, E).

Together these data demonstrate that following the exogenous addition of 5-ALA on day 1, a positive correlation was observed between the net photosynthetic rate and cell growth, biomass accumulation, fatty acid yields, and astaxanthin yields (Fig. 6A-D). Feeding these *H. pluvialis* cells with 5-ALA on day 1 can thus facilitate improved cell growth as a result of an enhanced net photosynthetic rate, thereby increasing astaxanthin and fatty acid production (Fig. 6E).

4. Conclusion

These results establish a novel approach to enhancing *H. pluvialis* biomass, astaxanthin, and fatty acid yields through the exogenous administration of 5-ALA during the early stages of growth. Adding 4 μM 5-ALA early during the cell growth can facilitate improved cell growth as a result of an enhanced photosynthetic activity, thereby increasing astaxanthin and fatty acid production. These results confirm the essential role of photosynthetic carbon assimilation in astaxanthin production and help in achieving cost-effective astaxanthin production in commercial cultivation of *H. pluvialis*.

CRediT authorship contribution statement

Qianqian Li: Investigation, Writing – original draft, Conceptualization, Methodology. **Fei Zhang:** Formal analysis, Software, Investigation. **Litao Zhang:** Supervision, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the Natural Science Foundation of Guangdong Province (2019A1515012066), the Science and Technology Program of Guangzhou (202102020839), and the Key Laboratory of Marine Ecological Conservation and Restoration, Ministry of Natural Resources/Fujian Provincial Key Laboratory of Marine Ecological Conservation and Restoration (EPR2022004).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.128319.

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